

The Role of Instrumental-methodical Developments with the Use of Synchrotron Radiation for Researching the Transformation of Nanostructural Parametres in Biological Objects

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Abstract

The major elements involved in instrumental and methodical developments for analysing the structural biology of tissues using synchrotron radiation (SR) from VEPP-3 and Siberia-2 storage rings of two Russian centres of collective using of SR at Novosibirsk and Mooscow were considered. Our basic concern was to study the mechanisms of structural and functional stability of the native and transformed human and animal tissues under various physicochemical influences. Possibilities of produced equipment and methods, as well as the experimental data on biological polymers having different degrees of order at the molecular or nanoscale level were presented.

Keywords

SR; VEPP-3; Siberia-2; WAXS; SAXS; Zoom Lenses

Introduction

X-ray stations and research methods with the use of SR for a new direction, the structural biology of tissues, namely, a the time-resolved small-angle X-ray diffractometry method named by us as "diffraction cinema", and methods of two-coordinate recording of different degree biological structures of order which allow carrying out noninvasive researches of the functional dynamics directly in a living tissue were developed and optimized (Vazina, Gadzhiev et al.; Gadzhiev, Gerasimov, Korneev et al.; Gadzhiev, Gerasimov, Gorbunova et al.; Gerasimov, Korneev et al.). One of the key goals of the present structural

biology is medical diagnostics that based on analyzing the characteristics of abnormalities at different levels of biological structures in the case of diseases and inborn pathologies caused by pathogenic factors of endogenous and exogenous nature. Every disease is connected with the changes in cell and/or tissue biochemistry with subsequent effects on the tissue structure, therefore the presence and the type of a disease can be determined by analyzing the tissue molecular structure. In other words, tissue can be used as a marker of the organism pathological states. Our basic concern is to study the mechanisms of structural and functional stability of the native and transformed tissues (including malignancies) under various physicochemical influences: temperature, humidity, mechanical load, and ionic selectivity (Vazina, Lanina et al.; Vazina, Budantsev et al.).

Thereupon, two aspects of SR use for tasks solution of tissue structural biology are determined: first, instrumental developments for research of biological polymers functioning mechanisms at molecular and nanosized scale are carried out, and secondly, X-ray diffraction methods under wide (WAXS) and small (SAXS) angles are developed. Weak scattering ability of biological tissues imposes excessively strict requirements on X-ray source intensity and the concentrations of investigated samples. Each type of the tissue is characterized by the specific set of fibrillar proteins with structural periodicity from 0,1 to 100

nanometers. The difference between functionally important structures is obvious in the characteristics and the regularity degree, as well as in time parameters of their structural dynamics. Large-scale X-ray diffraction and biological object spectral investigations exist due to the application of the unique experimental SR-based methods developed earlier (Vazina, Bras et al.; Aul'chenko, Vazina et al.).

SR is promising for application in structural biology. Due to its unique features (intensity, broad radiation spectrum and coherence) SR allows the acquisition of unique structural object information on objects with sizes ranging from fractions of a nanometer to several centimeters. This means that in living systems the functional range coverage is from single molecules, cells to tissues, and even organs, so that we can get complete information about the object.

For the decision of tissue structural biology problems SR was used in two Russian centres of collective use – in the Siberian and Moscow regions (Kulipanov; Belyaev et al.). X-ray stations were created on beam-lines of storage ring VEPP-3 (Siberian synchrotron and terahertz radiation centre SSTRC, Novosibirsk) and Siberia-2 (Kurchatov center nano-, bio-, information, cognitive and sociohumanity sciences and technologies NBICS, Moscow). Experimental technique and methods of studies were intended to carry out the following experiments: cross-striated skeletal muscle structural dynamics and structure research, normal and pathologic epithelial tissue samples; of natural and engineering silk construction study, and also high-frequency electrosurgical welding (HF-welding) influence on biological tissues.

Instrumental-methodical Development Results

The previous development results were used during X-ray station design and modernization, taking into account beam-lines features of SR of VEPP-3 and Siberia-2 storage rings. Several experimental equipment generations were created for biological nanostructures operational analysis (Aul'chenko, Baru et al.; Aul'chenko, Gadzhiev et al.; Ariskin et al.; Korneev, Sergienko et al.).

Figure 1 shows schemes and fragments of the X-ray station: a) - the structural scheme; b) - the generalized optical scheme; c) - a photo of block of station. Main optical planes are presented on the structural scheme; the path of X-rays in meridional and sagittal sections from the SR source (1) to its image on recording system of detector (2) is shown. Increase of intensity

and decrease of beam size image are provided by its focus on two orthogonally related planes.

The wide angular range recording of diffraction stream from the object (3) is required for carry out experiments. In modern recording conditions, it is achieved by alteration of sample – detector distance. There are two ways to meet the given requirement, first of which is to displace only the object (3) along the optical axis and to keep the distances between X-ray source (1), X-ray optical elements (4 and 5) and detector (2) invariable; while the other is to displace only the detector (2), but under this condition it's required to provide variable continuous with the focus on the X-ray stream.

The second way is more preferable for beam-lines conditions of SR source. Thus, radiation source (1) image variable focusing was carried out for different experiment requirements to meet the conjugate points conditions (where radiation source and its image are located), and input beam collimation system design into the diffraction pattern recording plane if necessary. The second way allows decreasing source image size and, increasing spatial resolution of diffraction pattern, accordingly. Thus, even focusing condition is provided in X-ray optical elements, named X-ray optical zoom lenses: the first element-monochromatic zoom lens (4); the second element-mirror zoom lens (5). X-ray stream focusing is carried out at the expense of the X-ray optical elements bend in the form of the circular cylinder with variable radius. Monochromatic zoom lens 4 works on Bragg diffraction principle from symmetrically cutoff crystal and provides two functions- required wavelength extraction with the given bandwidth and stream focusing in sagittal plane. Mirror zoom lens (5) consists of blocks with total external reflection 52 focusing mirrors and forms X-ray stream in meridional plane. The design for mirror sliding surfaces position correction in equatorial plane was carried out to allow asymmetrical element background lights avoidance along X-ray beam route. In this case, the followings are provided: the first mirror input into SR beam; other mirror setting relative to upper beam bound, passing under previous mirror; optimum sliding angles adjustment of mirrors to the beam and its focusing with SR source obtained from consecutive images superposition. Specialized background slits with independently controlled shutters were developed for X-ray optical elements background radiation interception. Main slits are installed in front of the zoom lens 4 and the object 3 (Figure 1b).

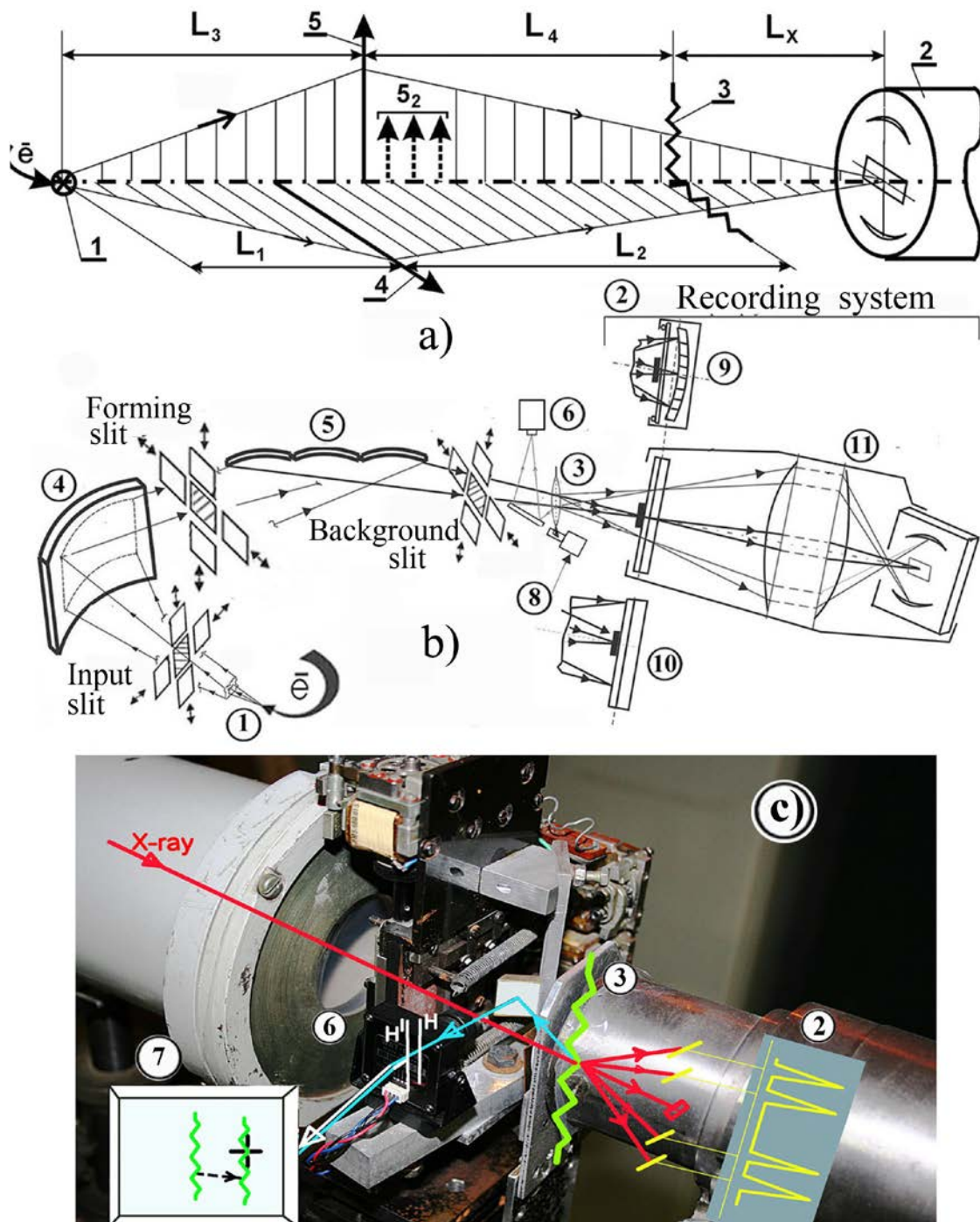


FIG. 1 SCHEMES AND FRAGMENTS OF THE X-RAY STATION

Crystal and mirrors arrangement reversed variant which offered by the authors and realized in the Siberian centre is presented on the scheme (Aul'chenko, Baru et al.). This variant allows solving three main problems – increase input beam aperture, decrease radiative and thermal load on mirrors which are the least resistant to X-ray influence, and fulfill conditions of main wavelength harmonic attenuation at the expense of optimal beam glancing angle to different density mirror surfaces selection.

Object visualization device (3) through chamber (6)

with monitor (7) on which there is SR-beam reference mark is created in last variant of the station. Object zone (3) is combined with the mark by means of positioning device (8) for studying.

At present, X-ray station on channel 1.3 a) of the storage device Siberia-2 has the following parameters: crystal Ge (111); wave length $\lambda = 0,16$ nm at $\Delta\lambda/\lambda = 10^{-3}$; the first mirror from fused quartz; distances between mirrors 0,35 m; $L_1=15,3$ m, $L_2=3,81$ m, $L_3=15,76$ m, $L_4=3,35$ m and $L_x=0,0035 \div 2,45$ m. Calculation results with the given parameters on the equations given in

(Korneev, Sergienko et al.), show that monochromatic and mirror zoom lenses should provide optical magnification gradual change in ranges: $V_m^X = 0,251^X \div 0,408^X$ and $V_3^X = 0,215^X \div 0,367^X$ respectively.

It is necessary to execute at least two conditions for developing X-ray optical scheme with the given parameters. The first one is to provide crystal and mirror bending radii in the range $R_m = 25,9 \div 36,2$ m and $R_3 = 1474 \div 2239$ m respectively. Secondly during mirror zoom lense adjustment, it is necessary to create conditions of beam optimum glancing angle to every mirror taking into account its focusing surface material. For example, when three-modular system ($L = 3,5 \times 10^{-3}$ m) is assembled and the first mirror is adjusted on the maximum input aperture (glancing angle 13 angular minutes), it's necessary to mount the mirrors that follow at the angles 13,7 and 14,9 angular minutes. Thereupon, either quartz mirrors should have coverage material of elements with large atomic weight or the first mirror should be adjusted onto a smaller glancing angle.

As known (Korneev, Shlektarev et al.), the crystal and mirrors are made as full-strength rectangular plates with t -thickness whose circular cylindrical surface is formed by torsion torque at the expense of the bending on four roller bearings, two of which are fixed and located on the operating surface sides. Two other movable bearings with L -distance between the centers are symmetrically installed at the element ends from the opposite side on a - equal distance from the first

bearings. To form X- ray optical element with optimal focal length, it is necessary to displace both bearings from the bent plate flat arrangement at the value δ_{np} , defined as

$$\delta_{np} = 0,08a(3L - 4a)(1/V + 1/U)\sin\tau + t[1 - 1/\cos[\arcsin L(1/V + 1/U)\sin\tau]] \quad (1)$$

where U , V are, respectively, the distances between the front butt of the focusing element and SR-source or between the front butt of the focusing element and the detector correspondingly; τ is stream glancing angle to element operating surface.

Movable support movement is carried out with the help of step motor drive actuator component. Its minimum discrete shift is called *zooming step*. SR-source image formation control is provided by optimal zooming step implementation, which depends on several X-ray optical scheme parameters. The most significant restrictions in our opinion, are connected with two conditions: for crystal this is monochromaticity degree $\Delta\lambda/\lambda$, which is determined by the studied object characteristics; for crystal and mirror this is depth of that is focal depth.

According to the given conditions, let us designate zooming step as $\Delta\delta(\Delta\lambda/\lambda)$, $\Delta\delta(dV/dR)$ respectively. Zooming step, calculated for curcular cylindrical single crystal boundary formation with a different cutoff angle α dependent on experiment conditions with the given diffracted beam monochromaticity degree $\Delta\lambda/\lambda$, which can be defined by the expression:

$$\Delta\delta\left(\frac{\Delta\lambda}{\lambda}\right) = \frac{0,666\left(\frac{\Delta\lambda}{\lambda}\right)a(4a - 3L)\cos 2\Psi \operatorname{tg}\Theta}{L\left\{\frac{\cos(\Theta \pm \alpha - \Psi)\left[0,5 - \frac{\operatorname{tg}(\Theta \pm \alpha - \Psi)}{A^2 \sin(\Theta \pm \alpha)}\left(\frac{\sin 2\Psi}{\sin(\Theta \pm \alpha)} - \cos[2\Psi - (\Theta \pm \alpha)]\right)\right]}{\sqrt{A^2 - 0,25 \sin^2(\Theta \pm \alpha - \Psi)}} - 1\right\}} \quad (2)$$

Where

$$A^2 = 0,25 + \frac{\sin\Psi}{\sin(\Theta \pm \alpha)}\left[\frac{\sin\Psi}{\sin(\Theta \pm \alpha)} - \cos(\Theta \pm \alpha - \Psi)\right] \quad (3)$$

$$\Psi = 0,5 \arcsin \frac{L \sin(\Theta \pm \alpha)}{U} \quad (4)$$

Θ is the central ray glancing angle on the SR-incident beam relative to crystallographic plane;

SR-source depth of field in the detection plane dV is determined by image size Z allowable increase, i.e. by the value $(Z + 2\delta)$ where δ corresponds to the recording equipment spatial resolution. Zooming step calculated from the given conditions can be defined by the

expression:

$$\Delta\delta(dV/dR) = \frac{\delta \cdot a \cdot (4a - 3L) \cdot \sin\tau}{3U \cdot (L \cdot \sin\tau - Z)} \quad (5)$$

X-ray stations were equipped with different recording systems (Figure 1): high-speed one-coordinate detector OD-3 (design INP SB RAS) (9); Fujifilm BAS-5000 analyzer (Japan) with image plates (10); two-

coordinate detectors (11) with cooled CCD-matrixes where images from X-ray fluorescent screens by means of optics (a system based on HS101H digital camera with fiber-optic plate (FOP) and with matrix S9737-02, Hamamatsu) or by means of optic fiber (SX-165 - Mar, USA) were transferred.

Method "diffraction cinema" with one-coordinate detectors was first developed in 1973 in the Siberian centre of SR to solve problems on structural transformation dynamics during biological functioning, during muscular contraction in the first place (Vazina, Gadzhiev et al.). Several versions of the given method with experiment control, received data acquisition and received data processing suite were developed (Aul'chenko, Bukin et al.). In the following years, modernization of the method "diffraction cinema" on different SR-sources was carried out. Experiments were carried out in dynamics, as well as in static mode, tracing temporal progress of object response on various physicochemical influences.

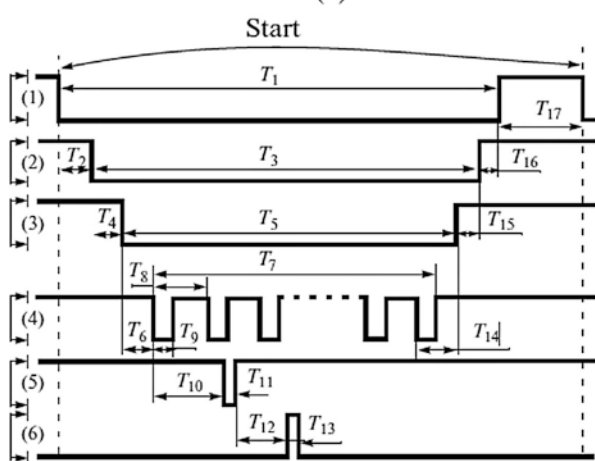
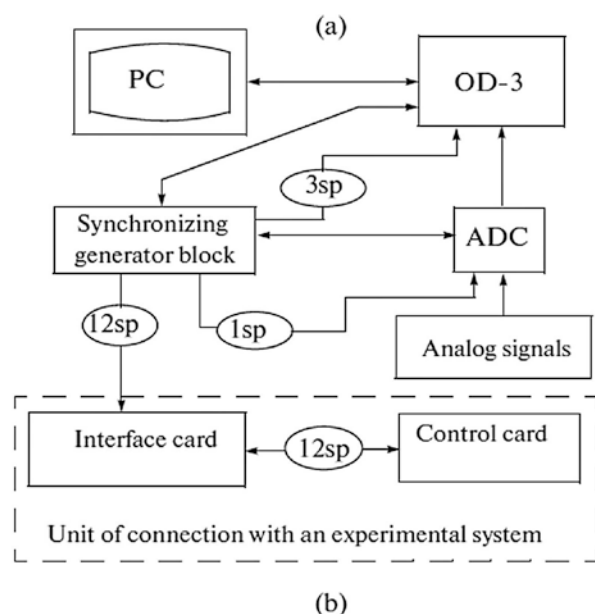


FIG. 2 BLOCK DIAGRAM (a) AND TIME DIAGRAM (b) OF SACR

To carry out investigations by the given method, the system for actuator control and recording of the X-ray and mechanical characteristics of different objects (SACR) has been developed. Its block diagram is depicted in Figure 2. With millisecond time resolution, the system allows measuring muscle physiological parameters during contraction: developed force, muscle length and diffraction reflection intensity changes that are conditioned by muscle molecular structure. In the experiment diffraction pattern (frame) of structure state set that corresponds to muscle contraction process successive stages were recorded. Measurement time is divided into a definite number of time intervals (frame durations), in each of which one measurement is taken. Time interval from the beginning of one measurement to the beginning of the next one is called "data storage cycle." Minimum frame duration is 1 μ s, which is limited by the available memory capacity (this capacity is programmed by a 32-byte number that is ≈ 4295 s for the given device).

In CRS-synchronizing generator unit and control card in a coupler of experimental X-ray complex (a) were developed in addition to the basic detector. Synchronizing generator unit can create time sequence up to 16 sync pulses (s/p) from which only four service sync pulses are used to synchronize detector itself and the others are used to control external devices. It should be noted that optical isolator (interface card) is used for all signals. S/p-sequence for the particular research object is recorded into synchronizing generator memory and can be performed in response to a PC-command. To investigate a new object, it's necessary to change control card.

The following s/p time sequence is recorded in the synchronizing generator unit: detector count control (cinema frame) – 3 s/p; ADC trigger control – 1 s/p; experiment external device control – 12 s/p. For sufficient statistics collection information is to be summed via multiple repetitions of muscle contraction cycles.

Experiment time diagram with the example of 6 element control: pump to transport physiological solution from the cuvette with the muscle (1); X-ray beam shutter (2); force sensor (3); DP-3 (4); muscle positive (5) and negative (6) electrostimulation device is shown in Fig. 2 b). After the start of one muscle contraction cycle the following device running times are provided: T_1 for pump; T_3 for shutter; T_5 for force sensor; T_7 is count time of DP-3 in one cycle and T_8 is count time of DP-3 in one frame.

Interpulse intervals: $T_2 \approx 2\div4$ s (shutter start after pump activation); $T_4 \approx 150\div200$ ms (force sensor start after shutter activation); $T_6 \approx 10$ μ s (DP-3 count start after force sensor activation); $T_{10} \approx 50$ ms (muscle excitation by stimulating impulse after OD-3 start); $T_{14} \approx 10$ ms (force sensor work completion after DP-3 count finite sync pulse); $T_{15} \approx 2\div5$ μ s (beam shutoff by the shutter after force sensor work completion); $T_{16} \approx 200\div300$ μ s (pump reverse start to fill cuvette with the solution after beam shutoff by the shutter); T_{17} is set experimentally (time when the muscle remains relaxed up to the next muscle contraction cycle). On the time scale, the sps durations are $T_9 \approx 5\text{--}15$ μ s (the OD-3 start) and $T_{11} = T_{13} \approx 1\text{--}5$ ms (positive and negative stimulations with the interval $T_{12} \approx 3\text{--}5$ μ s).

Experimental Results and Discussion

The experimental results which are received by diffraction SAXS/WAXS methods and characterize possibilities of instrumental and methodical provision in the SR beamlines for X-ray diffractive researches of the biological objects and other samples when using various systems of registration are presented in the given section on figure 3 - 8.

On Figure 3 the results of investigating the frog sartorius muscle obtained through the method "the diffraction cinema" are illustrated. On Figure 3 a) X-ray diffraction pattern and time dependence of intensity of the strongest meridional reflection of 14,3 nm contracted muscle initiated by single (1) and double (2) stimulations are presented.

In an active striated muscle, a short-lived (about 20 ms) structure state that is formed by the interaction of myosin and actin filaments is detected. The original hypothesis of the force generation mechanism on the basis of dynamic coupling principle of the nano-structures with different symmetries and incommensurable periods is proposed (Vazina). In Figure 3b) the time dependences of the equatorial reflections intensities during the isometric contraction initiated by double stimulation are shown. The time dependence of reflections intensities is illustrated from (10) and (11) planes, where thick and thin filaments in a hexagonal lattice are formed. In the normal state of a muscle (pos. 1), the maximum of the developed force coincides with the minimum of reflection (10), and the maximum of reflection (11) is observed earlier (≈ 20 ms). In the case of significant tiredness (pos. 2), strength is decreased more than 2 times while the reflections intensities remain almost unchanged. The second stimulus

applied in the relaxation phase does not change the behavior of reflection (11). However, when the optimal interpulse interval is selected and the second stimulus is applied in the phase of increasing tension, a muscle resuscitation process is observed (pos. 3) with respect to the sharply increased reflection (11) exceeding changes in reflection (10) in absolute magnitude. Thus, the second stimulus can be regarded as the tool of directional contraction behavior change in different phases of the active muscle state.

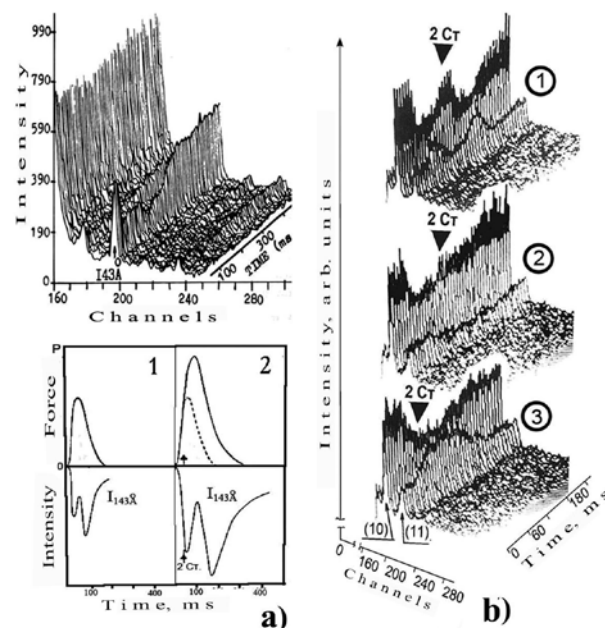


FIG. 3 X-RAY PATTERNS OBTAINED BY THE METHOD "THE DIFFRACTION CINEMA"

For definition of molecular and nanostructural configuration of biological tissue, where there is no strict ordering (crystallographic order) of high-molecular biopolymers two-coordinate detecting systems are formed and used, methods of X-ray diffraction under wide and small angles (WAXS/SAXS) are developed. For comparison, two diffraction patterns with different registers (a) obtained from silver behenate ($C_{22}H_{43}O_2Ag$) and the profiles of reflections intensities at the horizontal integration width (K bandwidth at $\lambda = 1.65$) are depicted in Figures 4 (a) and (b) respectively. The left-hand side X-ray pattern was obtained with SX-165 detector ($L = 290$ mm, $I = 125$ mA, and $T = 10$ s). On the right – the results with the system on the basis of ICX-249 matrix and a Hamamatsu FOP with a CsI scintillator ($L = 290$ mm, $I = 71$ mA, and $T = 20$ s). The observed reflections from the fundamental period $d_{001} = 58.4$ up to $d_{005} = 11.7$ characterize the recording system's sensitivity, which can be estimated, e.g., in the percentage ratio of peaks intensities. In the left-hand side diffraction

pattern, reflections localization makes up about 100, 65, 49, 35, and 33% in dynamic range of no less than 60 dB. In the right-hand part, a similar ratio makes up 100, 55, 40, 27, and 26%. In addition, the contrast sensitivity is smaller and the intensity of the last reflection is comparable with the observed noise level in the estimated dynamic range of about 20 dB.

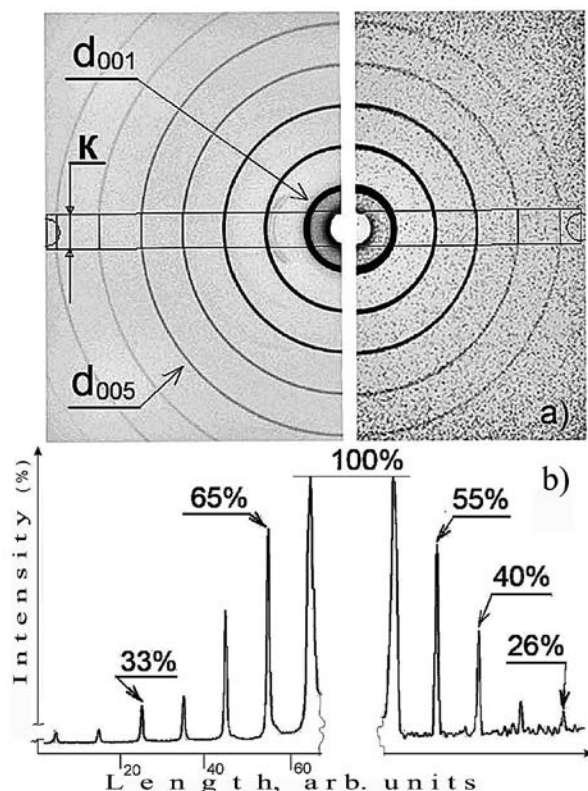


FIG. 4 X-RAY PATTERNS WITH DIFFERENT REGISTERS (a) OBTAINED FROM $C_{22}H_{43}O_2Ag$ AND THE PROFILES OF REFLECTIONS INTENSITIES (b)

In the given work intact and transformed epithelial human tissues at various stages of pathology were used as our objects of study (Figure 5). The special attention was given to the analysis of epithelial tissues producers (hair) that serve as markers of endogenous and exogenous effect, including not only environmental pollution, but also the impact of foodstuff, drugs, personal hygiene and cosmetic products (Aksirov et al.; Vazina, Bras et al.).

In Figure 5a) the combined X-ray patterns from hair tissues (above) and from a tumor tissue of the mammary gland (below) are presented. While in Figure 5b) the pattern in the range of wide angles is presented. The characteristic reflections are: a ring reflection 4,5 nm, caused by extracellular proteoglycan structures periodicity; the meridional reflection 6,7 nm and an equatorial reflection 9 nm, caused by intracellular keratin fiber. Similar diffraction patterns are obtained from various epithelial tissues of humans

and animals. A comparative analysis of obtained earlier X-ray patterns from different mucus samples and biological tissues showed that the $4.65 (\pm 0.15)$ nm identity period is a nanostructural invariant of giant proteoglycan molecules both of mucus and intercellular matrix of tissues. Structural hairs dynamics was studied during stretching (Figure 4c) and shows 6.7 nm meridional reflection distinct displacement towards small angles up to 8 nm (at a stretching about 50%). It is shown that the shift value remains unchanged along the whole length even of the longest hair (about 1 m). In this case, equatorial reflection at spacing of 4.5 nm remains unchangeable.

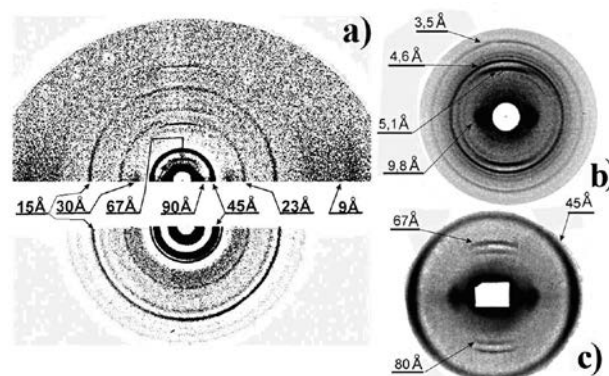


FIG. 5 X-RAY PATTERNS FROM HAIR TISSUES (ABOVE) AND TUMOR TISSUE OF THE MAMMARY GLAND (BELOW)

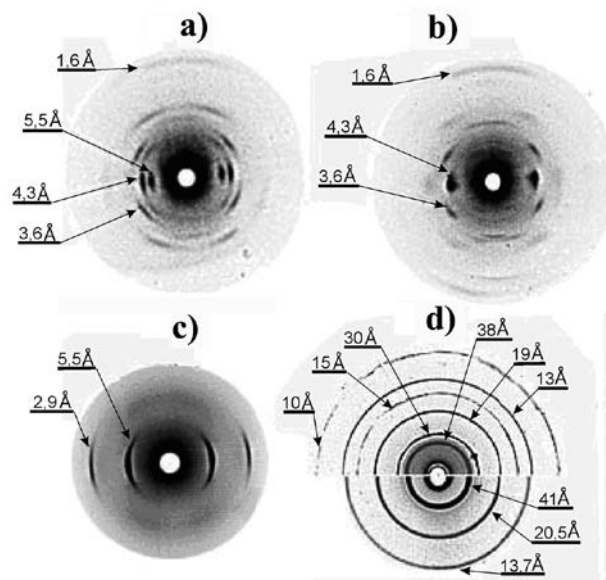


FIG. 6 X-RAY PATTERNS FROM OF NATURAL AND BIOENGINEERED SILK STRUCTURES

Silk protein structural dynamics systematic experimental studies are carried out last years. Silk proteins are localized as specific structures that function beyond an organism (Vazina). The studies were carried out on different natural and bioengineered natural silk structures (Figure 6) obtained from *Antheraea mylitta* (a) and *Bombyx mori* (b). Due to its high strength,

biocompatibility and biodegradability natural silk based biotechnological structures can be used as unique materials for biotechnological and medical purposes. Synthetic fibres X-ray picture is presented on Figure 6 c. Sharp intensive Debye rings series with 4.1, 2.05 and 1.37 nm periods is registered on superimposed bioengineered structures X-ray pictures-scaffolds (Figure 6 d) from *A. mylitta* silkworm (below). These rings are localized in positional ratio 3:2:1 typical for chain molecules linear periodicity property. Similar scaffolds X-ray picture from *B. mori* (above) shows much more Debye rings that can be grouped in two series with linear periodicity: 3.8, 1.9, 1.3 nm and 3.0, 1.5, 1.0 nm, and attributed to two chain molecules components that form 3D scaffold structure.

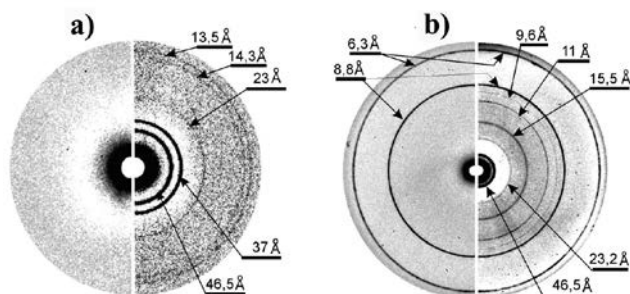


FIG. 7 X-RAY PATTERNS FROM INTESTINE TISSUES (a) AND SILK COCOON (b) IN THE NORM (LEFT PART) AND AFTER HF-WELDING (RIGHT PART)

Activities concerning synchrotron radiation application for living tissues nanostructural and molecular dynamics studies under high-frequency electric

welding have been initiated (Vazina, Vasilieva et al.). Intestine tissues (a) and silk cocoon (b) X-ray pictures in the norm (left part) and after HF-welding exposure (right part) are presented on Figure 7. HF-welding exposure revealed unique nanostructural order "birth" phenomenon-multidirectional fibrils self-assembly with nanostructural periodicity into intercellular ordered matrix. After welded seams X-ray picture HF-welding exposure intestine tissues are simply "filled" with sharp diffraction rings: the sharp Debye rings series with main period 4.65 nm is registered in nanoscale range from 10 to 0.4 nm. Nanostructural invariant 4.65 (± 0.15) nm attributed earlier as an identity period, determined by regular attachment of oligosaccharide chains to protein core of giant proteoglycan molecule of mucus and intercellular matrix of tissues (Vazina, Lanina et al.). New diffraction lines occurrence phenomenon after HF-welding was revealed on *Bombyx mori* silk cocoon samples: numerous thin Debye rings are registered in the range from 5 to 0.5 nm, determined by nanostructural periodicity. In this case molecular structure remained unchanged.

In FIG. 8, the generalized results at "DIKSI" station according to diffractometry SAXS/WAXS methods from objects are given: the silver behenate AgC_{22} and collagen of a rat tail tendon. It is shown that at monochromatic X-ray radiation with energy ~ 8 keV the reflections in 2Θ angular range from about 800 mrad to 2,5 mrad are registered.

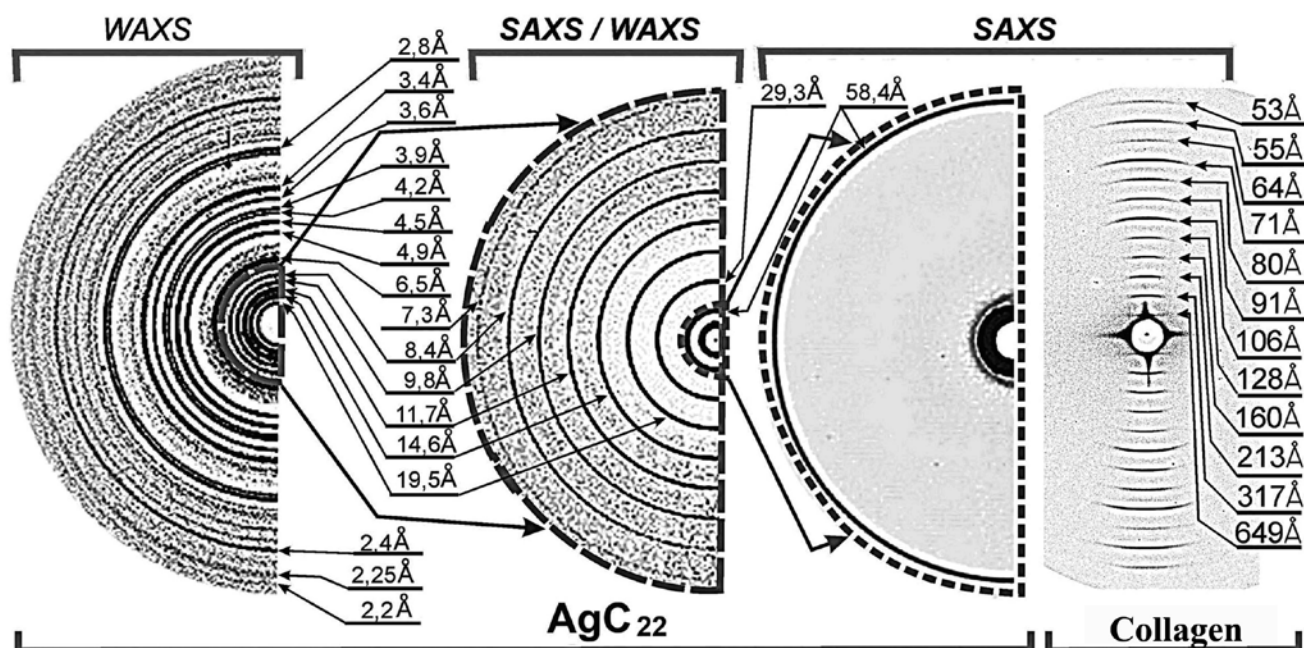


FIG. 8 GENERALIZED RESULTS AT "DIKSI" STATION ACCORDING TO DIFFRACTOMETRY SAXS/WAXS METHODS FROM AgC_{22} AND COLLAGEN

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